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**Bridging spatially segregated redox zones with a microbial electrochemical  
snorkel triggers biogeochemical cycles in oil-contaminated River Tyne (UK)  
sediments**

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## **Abstract**

Marine sediments represent an important sink for a number of anthropogenic organic contaminants, including petroleum hydrocarbons following an accidental oil spill. Degradation of these compounds largely depends on the activity of sedimentary microbial communities linked to biogeochemical cycles, in which abundant elements such as iron and sulfur are shuttled between their oxidized and reduced forms. Here we show that introduction of a small electrically conductive graphite rod (“the electrochemical snorkel”) into an oil-contaminated River Tyne (UK) sediment, so as to create an electrochemical connection between the anoxic contaminated sediment and the oxygenated overlying water, has a large impact on the rate of metabolic reactions taking place in the bulk sediment. The electrochemical snorkel accelerated sulfate reduction processes driven by organic contaminant oxidation and suppressed competitive methane-producing reactions. The application of a comprehensive suite of chemical, spectroscopic, biomolecular and thermodynamic analyses suggested that the snorkel served as a scavenger of toxic sulfide via a redox interaction with the iron cycle. Taken as a whole, the results of this work highlight a new strategy for manipulating biological processes, such as bioremediation, corrosion, and carbon sequestration, through the manipulation of the electron flows in contaminated sediments.

**Keywords:** Contaminated sediments, Iron cycle, Electrochemical snorkel, Oil spill remediation, Petroleum hydrocarbons, Sulfate reduction, Sulfide scavenging, Sulfur cycle

## **1. Introduction**

The release of thousands of tons of petroleum hydrocarbons (PHs) originating from anthropogenic activity affects the marine environment and causes severe ecological and economic damage (Bargiela et al., 2015). When an oil spill occurs, a variety of hydrocarbon removal strategies can be applied to minimize negative environmental impacts. Biological remediation methods are now widely used due to their lower environmental impact, adaptability to a range of contamination scenarios, and potential for full contaminant mineralization (Daghio et al., 2016). In confined hydrocarbon-rich environments, such as marine sediments, anoxic conditions prevail due to the excess of organic carbon that leads to rapid depletion of the most thermodynamically favorable electron acceptors (Bellagamba et al., 2016).

In anoxic sedimentary environments, the degradation of organic contaminants is usually stimulated by the addition of electron acceptors (e.g., oxygen, nitrate or sulfate) to provide microorganisms with more energetically favorable conditions for hydrocarbon oxidation (Meckenstock et al., 2004; Spormann and Widdel, 2000). Aerobic bioremediation is often favored over stimulation of anaerobic/anoxic contaminant degradation processes due to faster rates of hydrocarbon activation and removal (Viggi et al., 2015). In this context, a number of different approaches have been proposed to effectively deliver oxygen to contaminated anoxic sediments in order to accelerate hydrocarbon biodegradation (Genovese et al., 2014). However, rapid abiotic consumption of oxygen by reaction with reduced chemical species (e.g.,  $\text{Fe}^{2+}$ ,  $\text{S}^{2-}$ ) and difficulties in controlling the rate of oxygen release limit the practical application of these approaches.

An alternative approach to overcoming electron acceptor limitation in oil-contaminated sediments is the use of bioelectrochemical systems (BES) (Lovley and Nevin, 2011;

Wang and Ren, 2013). BES employ solid-state electrodes to directly or indirectly stimulate and control microbial metabolism. Stimulation of microbial activity is driven by the ability of “electro-active bacteria” (EAB) to exchange electrons with the electrodes, which can serve as electron acceptors or donors in their energy metabolism (Borole et al., 2011). Recent, laboratory-scale studies have demonstrated that BES-based technologies can be successfully applied to remove hydrocarbons from oil-contaminated sediments (Bellagamba et al., 2016; Daghighi et al., 2016; Morris et al., 2009; Yan and Reible, 2014).

The concept of bioelectrochemical bioremediation to accelerate hydrocarbons biodegradation in anoxic marine sediment was introduced recently (Viggi et al., 2015). The system was based on an “Oil-spill Snorkel” consisting of a rod of conductive material (i.e., an electrode) positioned to create an electrochemical connection between the anoxic contaminated sediment and the oxic overlying water. In principle, the snorkel could take advantage of the capability of EAB to anaerobically oxidize hydrocarbons with a carbon electrode, deployed in the sediment, serving as a respiratory electron acceptor. The electrons travel from the bottom of the snorkel buried in the sediment (anode) to the upper part of the snorkel immersed in the overlying oxic water (cathode) where oxygen is reduced to water, effectively spatially separating the oxidation of the electron donor (hydrocarbons) from the reduction of the terminal electron acceptor. Collectively, the results of this preliminary study confirmed the potential of the snorkel to accelerate biotic and abiotic oxidative reactions taking place within the sediment, as documented by a 1.7-fold increase in the cumulative oxygen uptake and 1.4-fold increase in the cumulative CO<sub>2</sub> evolution, in microcosms containing snorkels compared to snorkel-free controls. Accordingly, the initial rate of petroleum hydrocarbon biodegradation was also substantially enhanced (Viggi et al., 2015). Despite these

promising findings, the fundamental mechanisms underlying the observed enhancement of hydrocarbon degradation could not be identified and the overall impact of the microbial electrochemical snorkel on key sediment biogeochemical cycles were not elucidated. To gain a deeper understanding of these critical factors, further oil-spill snorkel experiments were conducted using petroleum-contaminated estuarine sediments from the River Tyne (UK) which had been previously reported to harbor active sulfate-reducing, and syntrophic methanogenic hydrocarbon-degrading microbial communities (O'Sullivan et al., 2015; Sherry et al., 2014).

## **2. Materials and Methods**

### *2.1 Experimental setup*

The experiments described below are hereafter referred to as oil-spill snorkel experiments. The estuarine sediments used in this study were obtained from the River Tyne (UK). Sediment samples were subject to four treatments; 1) oil-supplemented sediment containing 3 graphite rods, termed “snorkel”, 2) sediment with no oil amendment containing 3 graphite rods, termed “no oil snorkel”, 3) oil-supplemented sediment without graphite rods, termed “control”, and 4) sediment with no oil amendment or graphite rods, termed “no oil control”. The oil containing treatments were amended with Danish Underground Consortium (DUC) light crude oil to a final concentration of approximately 20 g/kg. To prepare oil contaminated sediment, the sediment was divided into 4 equal portions; one part was thoroughly mixed with oil previously dissolved in hexane. The hexane was evaporated by air-drying the sediment. Finally, the air-dried contaminated sediment was mixed, under nitrogen, with the remaining three parts of the sediment. This procedure has been used in order to achieve

a homogenous contamination of the sediment while preserving a large fraction of the sediment microbial communities (Viggi et al., 2015). Microcosms containing oil-supplemented sediment or unamended sediment were prepared in 100-mL glass cylinders. Each cylinder contained (starting from the bottom) ~90 grams of sediment, a layer of Norit® granular activated carbon (10 g, serving as high surface area oxygen reduction catalyst) (Zhang et al., 2009) and 25-30 mL of synthetic brackish medium (Sea Salts, Sigma Aldrich, diluted 1:4). For the “snorkel” and “no oil snorkel” treatments, three graphite rods (the snorkels) were inserted vertically through the layers of different materials to create an electrochemical connection between the anoxic sediment and the oxygenated overlaying water. Replicate microcosms were prepared for each treatment and were sacrificially sampled after incubation for 0, 118, 175, 286 and 466 days. The microcosms were statically incubated in the dark at  $20 \pm 1$  °C. After 0, 118, 175, 286 and 466 days of incubation, one cylinder from each treatment was sacrificed, hydrocarbons were extracted from the sediment and analyzed by GC-MS; the aqueous phase (overlying water) was analyzed by ion chromatography (IC) for quantification of anions and the gas phase (i.e., gas pockets accumulating within the sediment) was analyzed by GC-TCD for quantification of methane.

## 2.2 Analytical methods

Methane in the gas pockets was quantified by gas chromatography (GC). Gas samples (50 µL) were taken from voids in the sediment that occurred in some treatments using a gas-tight syringe (Hamilton, Reno, NV, USA) equipped with an 8-cm long needle. Gas samples were analyzed with a Perkin-Elmer Auto System gas chromatograph [stationary phase: stainless-steel column packed with 60/80 Carboxen™ 1000 molecular sieve



(Supelco, USA); carrier gas: N<sub>2</sub> at 20 mL/min; oven temperature: 150 °C; injector temperature: 200 °C; thermal conductivity detector (TCD) temperature: 200 °C].

Anions in aqueous phase samples were quantified by ion chromatography (IC). Samples were filtered (0.22 µm pore size) and injected into a Dionex DX-100 system (Dionex Corp., Sunnyvale, CA) ion chromatograph (column: IonPac AS14; eluent: sodium carbonate 3.5 mM / sodium bicarbonate 1.0 mM solution). Quantification of total petroleum hydrocarbons (TPH) in sediment samples was performed by GC-MS. In brief, sediment samples were air dried and extracted with a Dionex ASE 200 (Dionex Corp., Sunnyvale, CA) using an acetone:hexane (1:1 v/v) mixture at 100 °C and a system pressure of 1500 psi. The solvent extract was evaporated under a stream of nitrogen and re-dissolved in 10 mL of an *n*-heptane containing *n*-dodecane (*n*-C<sub>12</sub>) and *n*-tetracontane (*n*-C<sub>40</sub>), each at 10 mg/L, as markers for the GC analysis. To purify the hydrocarbon extract (i.e., remove polar compounds), it was percolated through a solid phase extraction cartridge filled with Florisil® and anhydrous sodium sulfate (Chromabond® Na<sub>2</sub>SO<sub>4</sub>/Florisil®, 6 mL polypropylene columns, 2g/2g). A sample (1 µL) of the purified hydrocarbon extract was then injected (in pulsed split-less mode) into a GC-MS (Perkin Elmer Clarus 680/600; column: HP-5 MS (Agilent) 30 m, ID 0.25 mm, 0.25 µm film thickness; carrier gas: helium at 1 mL/min; injector temperature: 280 °C; oven temperature program: initial Temp 40 °C, 18°C/min to 250°C, 10 °C/min to 280 °C, hold for 17 min; MS-scan 30-600, 2-32 min). The TPH amount was determined by summing the unresolved and resolved components eluted from the GC capillary column between the retention times of *n*-C<sub>12</sub> and of *n*-C<sub>40</sub>, using solutions of diesel motor oil and diesel mineral oil in hexane as calibration standards (concentration range 0.15–2 g/L).

### 2.3 Microbial Community Analysis with Next Generation Sequencing

Thirty 16S rRNA amplicon libraries were generated representing communities from the sediment used to prepare the microcosms prior to incubation, which were either treated with oil or left untreated (Time 0). The microbial communities were also characterized in samples from sediments from the snorkel, control, no oil snorkel, and no oil control treatments following 175 and 286 days of incubation (n=3 for all). The microbial community analysis was conducted as follows.

### 2.3.1 DNA extraction

Extractions were performed in triplicate from sediment samples (~400 mg) using a PowerSoil® DNA Isolation Kit (Mo Bio Laboratories Inc., USA) with a ribolyser (FastPrep-24, MP Biomedicals, USA). Procedural blanks were performed to ensure extracts remained contamination-free throughout the extraction procedure. DNA extractions were carried out according to the manufacturer's instructions with the following minor modifications: the ribolyser was used at a speed of 6 m/s for 40 s for homogenisation and cell lysis. DNA extracts were stored at -20°C until further use.

### 2.3.2 PCR amplification 16S rRNA genes and PCR product purification

The variable V4/V5 region of the 16S rRNA gene was amplified using the degenerate primers, 515F (GTG-NCA-GCM-GCC-GCG-GTA-A) and 926R (CCG-YCA-ATT-YMT-TTR-AGT-TT) (Quince *et al.*, 2011). In silico testing of the primer set against the SILVA SSU 128 Ref NR database using TestPrime 1.0 (Klindworth *et al.*, 2013) returned values of 88.2%, 85.8% and 85.7% coverage against the Bacteria, Archaea and Eukarya domains respectively. DNA extracts were diluted (1/10) to reduce the levels of inhibitory contaminants to prevent PCR inhibition (Head, 1999). PCR reactions contained NH<sub>4</sub> buffer (50mM), 0.5 mM dNTPs (Invitrogen), 500 nM each primer (ThermoScientific), 2.5

U of *Taq* polymerase (Bioline), and 1 µl template DNA in a 20-µl volume. PCR conditions were initial denaturation (94°C, 4 min) followed by 30 cycles (95°C, 1 min; 55°C, 0.45 min; 72°C, 1 min) and a final extension (72°C, 10 min) in a thermal cycler (Techne TC-512, Bibby Scientific Limited). PCR products were analyzed by gel electrophoresis with 1% (w/v) agarose gel in 1 x TAE buffer (Tris-acetate-EDTA buffer; 40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.3). Electrophoresis was performed at 100 V for 45 min. Gels were stained with ethidium bromide and visualized with a BioSpectrum Imaging System with VisionWorks LS software (UVP, Cambridge, UK). PCR products were purified with Agencourt AMPure XP PCR purification kit (Beckman Coulter).

### *2.3.3 DNA quantification and Ion Torrent DNA sequencing*

Purified DNA was quantified on a Qubit 2.0 Fluorometer (Invitrogen) with a Qubit dsDNA high sensitivity assay kit (Invitrogen). The final concentration of DNA was adjusted to 100 pM, and equimolar concentrations of DNA from all samples were pooled. The pooled amplicon library was sequenced on an Ion Torrent Personal Genome Machine (Life Technologies). Briefly, the library was diluted (26 pM) and emulsion PCR performed on a OneTouch2 instrument with an Ion PGM Template OT2 400 kit according to the manufacturer's instructions (Life Technologies). Beads with bound template DNA were purified on a OneTouch ES system (Life Technologies). Following enrichment, the beads were loaded onto a PGM 316 chip and sequenced in accordance with the manufacturer's instructions.

### *2.3.4 Data analysis*

Raw sequence reads were retrieved using the Torrent Suite Software V4.0 (Life Technologies). Sequence reads with a modal length of 428 bp were analyzed in QIIME

(Caporaso *et al.*, 2010a). Sequences were assigned to samples based on their unique barcodes and simultaneously filtered to remove reads with no corresponding barcode, reads without the correct primer sequence and poor quality reads (those with a quality value of < 20 were discarded). Operational taxonomic unit (OTU) classifications were performed using UClust (Edgar, 2010), with an OTU threshold defined at 97% sequence identity. OTUs were first clustered open reference against the Greengenes 16S rRNA core alignment (DeSantis *et al.*, 2006) and then clustered de novo. Taxonomy was assigned using RDP Classifier (Wang *et al.*, 2007) and sequences aligned using PyNAST (Caporaso *et al.*, 2010b). Chimeric sequences were identified with ChimeraSlayer (Haas *et al.*, 2011) and removed before subsequent analysis. The average number of reads in individual binned libraries after filtering was 25,868 with a range from 11,214 to 139,159 reads. Libraries were rarefied to 11,214 reads for comparative analysis. Core diversity analysis was subsequently performed in QIIME v1.8 to provide a comparative analysis of the microbial communities between samples. BIOM table data, consisting of OTU counts per sample, were imported into Microsoft Excel to determine the average percentage relative abundance (n=3 for all treatments, except T0 where n=6). Phylum and class level comparisons were performed at a level of  $\geq 0.2\%$  relative abundance. Abundant taxa for further investigation were determined as those sequences with  $\geq 1\%$  relative abundance. The six amplicon libraries generated from the initial sediments (Time 0) with and without oil were very similar (Pearson correlation at the genus level  $R^2 \geq 0.957$ ,  $p \leq 0.01$ ), these were therefore treated as replicate samples giving n=6 for the Time 0 samples. Sequences have been deposited in the NCBI's Short Read Archive (SRA) under BioProject PRJNA376663.

## 2.4 Catalyzed Reporter Deposition-Fluorescence In Situ Hybridization (CARD-FISH) analysis

Sediment samples and biofilms growing on the surface of the graphite rods (i.e., the snorkels) were taken for CARD-FISH analysis of the microbial communities. In brief, approximately 1 g of sediment was fixed in formaldehyde (2% v/v final concentration) and cells were extracted from sediment particles as described previously (Barra Caracciolo et al., 2005). The detached cells were filtered through 0.2 µm polycarbonate filters (Ø 47 mm, Millipore) by gentle vacuum (<0.2 bar) and stored at -20°C until use. To remove the biomass from the biofilm formed on the graphite rods, the surface of the electrode was gently scraped with a sterile spatula. The detached biomass was collected in PBS buffer containing 2% v/v formaldehyde, and filtered as described above. CARD-FISH was carried out following a previously published protocol using probes targeting total *Bacteria* (EUB338 I,II,III), and *Deltaproteobacteria* (DELTA495a,b,c) (Fazi et al., 2008). Probes, labeled with horseradish peroxidase (HRP), were purchased from BIOMERS (<http://www.biomers.net>). Probe sequences and hybridization conditions were as reported in probeBase (<http://www.microbial-ecology.net/probebase>). DAPI (4',6-diamidino-2-phenylindole) staining was performed to determine total cell numbers, from which the relative abundances of each targeted bacterial population was calculated. Total cell counts were performed at the end of the CARD-FISH hybridization procedure by mounting the samples in Vectashield Mounting Medium with DAPI (Vector Labs, Italy). At least 20 randomly selected microscopic fields for each sample were analyzed to enumerate the cells by microscopic analysis. Slides were examined by epifluorescence microscopy (Olympus, BX51) and the images were captured with an

Olympus F-View CCD camera and images were processed with Cell<sup>F</sup> software (Olympus, Germany).

## 2.5 *geneCARD-FISH assay*

GeneCARD-FISH assays were conducted on small sections of filter (see section 2.3) according to a previously described protocol (Matturro et al., 2016), as detailed in the following paragraphs.

### 2.5.1 *Polynucleotide probe design and synthesis*

Polynucleotide probes targeting the gene encoding the alpha subunit of alkylsuccinate synthase (*assA*) were synthesized using a PCR DIG Probe Synthesis Kit (Roche, Italy) following the manufacturer's instructions. A cloned *assA* gene, obtained from a hydrocarbon-degrading sulfate-reducing enrichment culture (Aitken et al., 2013), was used as DNA template. Primers *assA2F* and *assA2R* (Aitken et al., 2013) were used for PCR amplification. PCR Dig-labeled amplicons were purified with QIAquick PCR Purification Kit (Qiagen, Italy) and run on a 2% agarose gel to check the incorporation of DIG molecules. The concentration of purified DIG-labeled polynucleotide probe was quantified (NanoDrop ND-2000, Italy) and aliquots (10 ng/μL) were stored at - 20°C and employed during the hybridization step of the geneCARD-FISH assay.

### 2.5.2 *Hybridization*

Before hybridization, cells were pretreated with lysozyme and proteinase K as previously described (Matturro et al., 2016). Filters were transferred to 100 μL of pre-hybridization buffer HB-I containing the following compounds: 0.25 mg/mL of thymus DNA, yeast RNA 0.25 mg/mL, formamide 50%, Saline Sodium Citrate SCC 5X, dextran

sulfate 10%, SDS 0.1%, EDTA 20 mM, blocking reagent 1% (molecular biology reagents, Sigma Aldrich, Italy) and incubated at 46°C for 1.5 h. Filters were then transferred to a fresh tube containing 100 µL of HB-I and DIG-labeled polynucleotide probe (final concentration 0.5 ng/µL) and then incubated in a thermocycler with the following cycles: 70°C for 25 min (DNA denaturation) and 46°C overnight (Dig-labeled probe hybridization). After hybridization, filters were immersed in washing buffer WB-I (SSC 10X, SDS 0.1%) for 30 min at 48°C and then transferred into washing buffer WB-II (SSC 0.1X, SDS 0.1%) for 90 min at 48°C.

### *2.5.3 Immunochemical probe detection*

Filters were incubated in an antibody buffer AB-I for 45 min at room temperature. Buffer AB-I consisted of a Western Blocking reagent solution 1% (Sigma Aldrich, Italy) in phosphate buffered saline, 1X PBS (145 mM NaCl, 1.4 mM NaH<sub>2</sub>PO<sub>4</sub>, 8 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.4). Filters were then immersed into an AB-I buffer solution containing the antibody anti-DIG (Anti-Digoxigenin-POD Fab fragments, Sigma Aldrich, Italy) to a final concentration of 0.3U/mL for 90 min at room temperature. After incubation, filters were washed for 10 min in fresh AB-I buffer at room temperature. Filters were then washed 10X PBS solution for 20 min at room temperature.

### *2.5.4 Fluorescent signal amplification*

After the immunochemical reaction, filters were placed in the substrate mix with dye-tyramide for 15 min at 37°C and then washed in 1X PBS for 30 minutes at room temperature, in water for 1 min and finally in 96% ethanol for 1 min. The filters were air-dried, placed on a microscope slide and stained with DAPI (4,6-diamidino-2-phenylindole) to determine total cell numbers in the samples.

319

#### 320 2.5.5 Microscopy analysis

321 Hybridized cells carrying the *assA* gene were visualized by epifluorescence microscopy  
322 (Olympus, BX51) and quantified by counting fluorescent cells (at least 100 cells per  
323 grid) on random grids on filter sections. Quantitative data were expressed as cell  
324 numbers per g dry weight of sediment.

325

#### 326 2.6 X-Ray Photoelectron Spectroscopy of graphite electrodes

327 The surface chemical composition of the graphite rods was determined by using X-ray  
328 photoelectron spectroscopy (XPS). XPS measurements were performed in a VG Escalab  
329 MkII spectrometer (VG Scientific Ltd, East Grinstead, UK) with a 5-channeltron detection  
330 system and an unmonochromatized radiation source of Al K $\alpha$  (1486.6 eV). The  
331 electrostatic lenses were operated in the selected-area mode A3x12, providing  
332 photoelectron collection from the sample area with a diameter of about 3 mm. The  
333 binding energy (BE) scale was calibrated by using the C1s peak of graphite at BE = 284.6  
334 eV. All the spectra were acquired at the pass energy of 50 eV. Spectroscopic data were  
335 processed using Advantage v.5 software, using a peak-fitting routine with Shirley  
336 background and Scofield sensitivity factors for elemental quantification.

337

338

### 339 3. Results and discussion

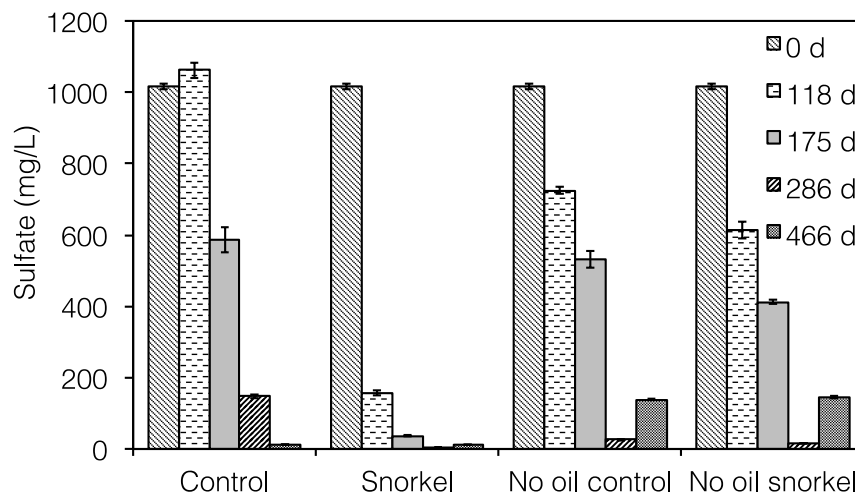
#### 340 3.1 Sulfate reduction

341 Sulfate is one the most abundant electron acceptors in anoxic marine and brackish  
342 sediments and typically plays a critical role in the oxidation of organic matter in pristine  
343 and contaminated sediments. Sulfate concentration in the snorkel treatments and



control microcosms was monitored following incubation for 0, 118, 175, 286, and 466 days (Figure 1). In the oil-supplemented control with no snorkel ("control"), sulfate concentration remained nearly constant during the initial 118 days of incubation (Figure 1); thereafter, sulfate gradually decreased over time and was almost completely depleted by day 466 (Figure 1). By contrast, significantly greater initial sulfate reduction rates were observed in the oil-supplemented snorkel-containing sediments (two-tailed t-test,  $p < 0.005$ ), with over 85% of the initial sulfate (i.e., approximately 1,000 mg/L) consumed by day 118 (Figure 1). Notably, sulfate reduction was observed also in microcosms that were not supplemented with oil, being most likely fuelled by the indigenous organic carbon in the River Tyne sediment. Also in this case, the snorkel accelerated (though to a lower extent) the removal of sulfate, with no-oil snorkel treatments displaying a 35% greater cumulative sulfate removal compared to the no-oil controls by day 118 ( $p = 0.06$ ), and a 25% greater cumulative removal by day 286 ( $p = 0.005$ ) (Figure 1). Notably, by comparing sulfate concentrations in the control and in the no oil control experiments, it is apparent that, in the absence of the snorkel, the presence of oil slightly inhibited the activity of sulfate reducing microorganisms in the River Tyne sediments (Figure 1).

A recent study (Daghio et al., 2016), suggested that an electrode potentiostatically poised at an oxidative value of +300 mV vs. SHE, was capable of indirectly stimulating the sulfate-driven anaerobic oxidation of toluene, through the electrochemical scavenging of hydrogen sulfide, a toxic end product of dissimilatory sulfate reduction (Reis et al., 1992). This hypothesis was supported by the accumulation of elemental sulfur on the surface of the poised carbon-based electrode.

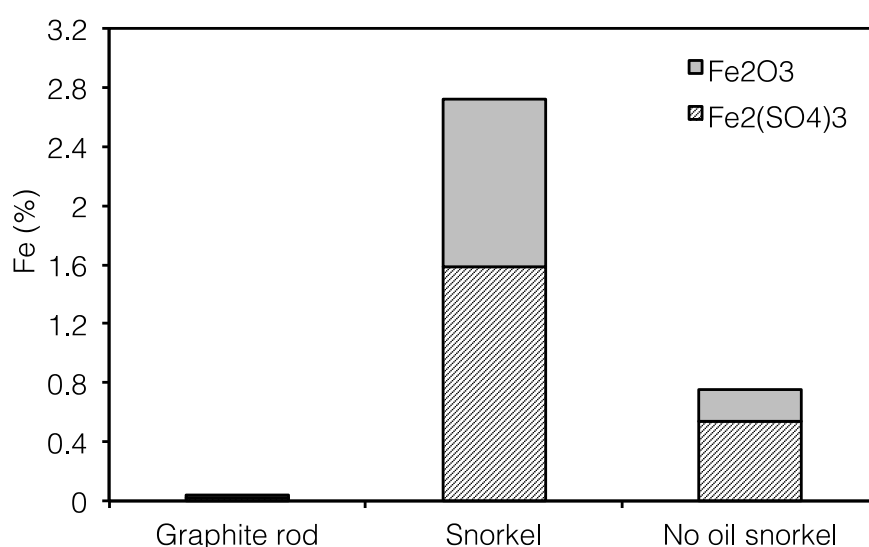


**Figure 1.** Concentration of sulfate in the snorkel experiments and controls throughout the experimental period. Error bars represent the standard error of replicate samples.

### 3.2 XPS analysis of graphite electrodes

To test the assumption that the enhancement of sulfate reduction may be due to the electrochemical scavenging of sulfide, at the end of the study (day 466), the surface of the graphite rods (from the snorkel and the no oil snorkel treatments) were analyzed via XPS. For comparative purposes, an identical pristine graphite rod was also analyzed. Interestingly, XPS analyses did not reveal the presence of elemental sulfur in any of the snorkel treatments. However, substantial amounts of oxidized iron species, namely  $\text{Fe}_2(\text{SO}_4)_3$  and  $\text{Fe}_2\text{O}_3$ , were detected on the surface of the snorkels (Figure 2). Notably, when the rods were extracted from the sediments, prior to being analyzed by XPS analysis, they appeared to be covered by reddish precipitates, providing a further qualitative indication of the occurrence of oxidized iron species. Importantly, the abundance of  $\text{Fe}^{3+}$  species in the oil-supplemented snorkel (2.8 atomic %) was 4-fold higher than in the no-oil snorkel (0.7 atom%) (Figure 2), raising the

intriguing possibility that the accumulation of iron precipitates was correlated with the rate and extent of observed sulfate reduction. Accumulation of oxidized iron species on the surface of a conductive snorkel deployed in crude oil marine sediment was also observed in a previous oil-spill snorkel experiment (Viggi et al., 2015).

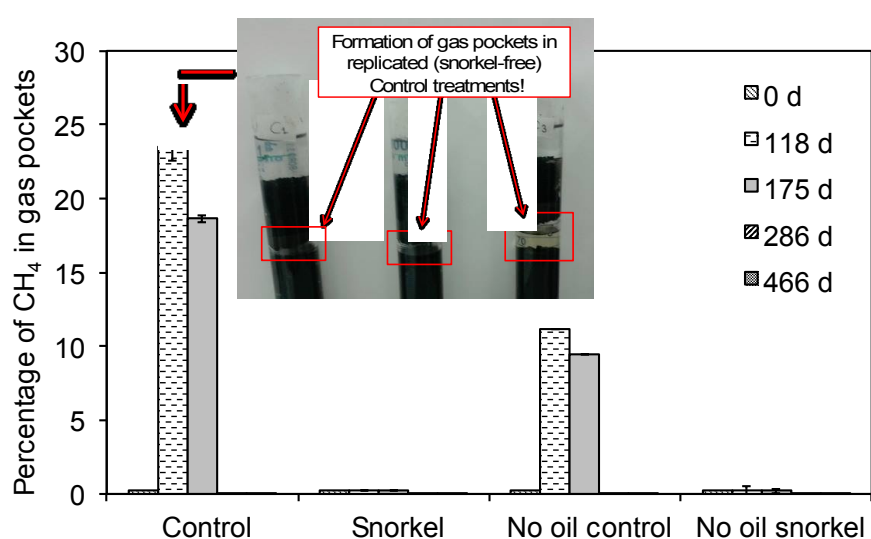


**Figure 2.** Percentage of Fe species on the surface of oil-supplemented and no oil snorkel experiments, and in untreated graphite rods.

### 3.3 Gas production

The presence of snorkels markedly affected gas production and methanogenic activity in River Tyne sediments. Indeed, gas pockets containing methane (up to 25% vol/vol) accumulated within the sediment in control microcosms, which did not contain the snorkel (Figure 3). By contrast, negligible gas production occurred in oil-supplemented and no-oil snorkel treatments. Although the total cumulative volume of gas produced in each treatment could not be precisely determined, the higher percentage of methane observed on days 118 and 175 in the gas pockets occurring in oil-supplemented controls

relative to the no-oil controls, provides an indication that at least some of the produced methane might derive from degradation of oil constituents, with the remainder likely deriving from the degradation of the organic matter already present in the sediment. In fact, methanogenic crude oil-degrading capacity has already been reported in the River Tyne sediments (Gray et al., 2011; Jones et al., 2008). In both oil-supplemented and no-oil controls, gas production peaked on day 118 and became nearly undetectable by day 286 (Figure 3).



**Figure 3.** Percentage (% vol/vol) of methane in gas pockets formed in snorkel-free control experiments. Error bars represent the standard error of replicate samples. The inset photo illustrates the gas pockets seen in snorkel-free sediments.

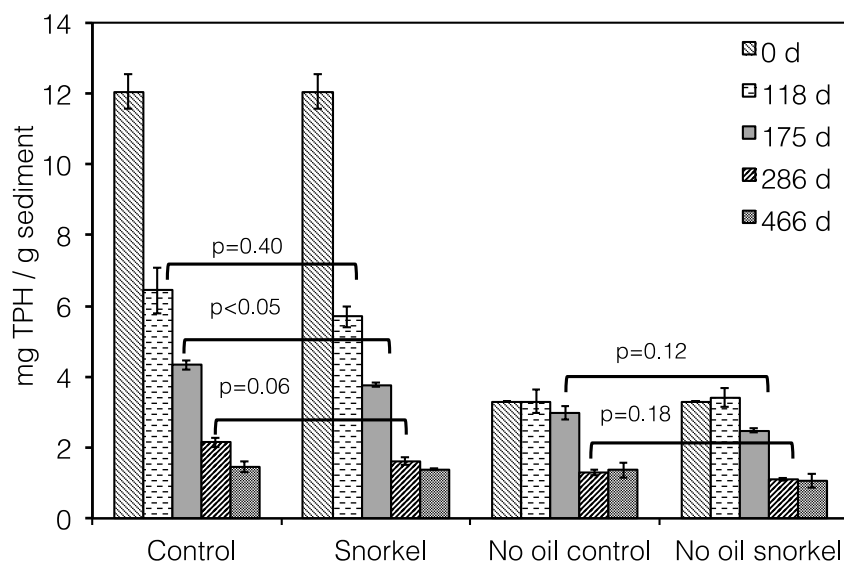
A large number of studies focusing on the anaerobic decomposition of organic matter in both freshwater and marine environments have stressed the importance of sulfate reduction and methanogenesis (Kuivila et al., 1989; Lovley and Klug, 1986; Oremland and Polcin, 1982; Oremland and Taylor, 1978). Thermodynamic and kinetic reasoning

suggests that sulfate-reducing bacteria can outcompete methanogens due to the higher energy yield of sulfate reduction to sulfide driven by H<sub>2</sub> (or acetate) relative to the corresponding reduction of carbon dioxide to methane (or acetate disproportionation to methane and carbon dioxide), as well as to the higher growth rate of sulfate reducers on these substrates, compared to methanogens (Conrad et al., 1986). Here, in microcosms containing the snorkel, sulfate-reducing bacteria almost completely outcompeted methanogens. By contrast, in microcosms not containing the snorkel, sulfate reduction (though at lower rate than in the snorkel experiments) and methanogenesis occurred simultaneously, suggesting the two metabolic processes are competitive yet not mutually exclusive (Paulo et al., 2015).

#### *3.4 Crude oil biodegradation*

In oil-supplemented microcosms, the removal of TPH appeared to be accelerated (up to 30% greater removal by day 175,  $p < 0.05$ ) by the presence of the snorkel (Figure 4). These findings are consistent with the results of a previous microcosm study carried out using a different sediment contaminated with a different oil, hence indicating that the snorkel-induced stimulatory effect on hydrocarbon biodegradation was reproducible in sediments from distant geographical locations (Viggi et al., 2015). Consistent with the industrial setting from which the River Tyne sediments were obtained, low levels of hydrocarbons were detected in the microcosms, which were not specifically amended with the DUC crude oil (Figure 4). In these microcosms, TPH concentration decreased over time to a similar extent both in the no oil control and in the no oil snorkel treatments. No stimulatory effect of the snorkel on removal of indigenous hydrocarbon contamination was apparent, possibly due to the fact that the indigenous hydrocarbons in the River Tyne sediment resulted from chronic

contamination and were probably less available to the hydrocarbon-degrading organisms present, compared to freshly spiked hydrocarbons in the oil-supplemented treatments.

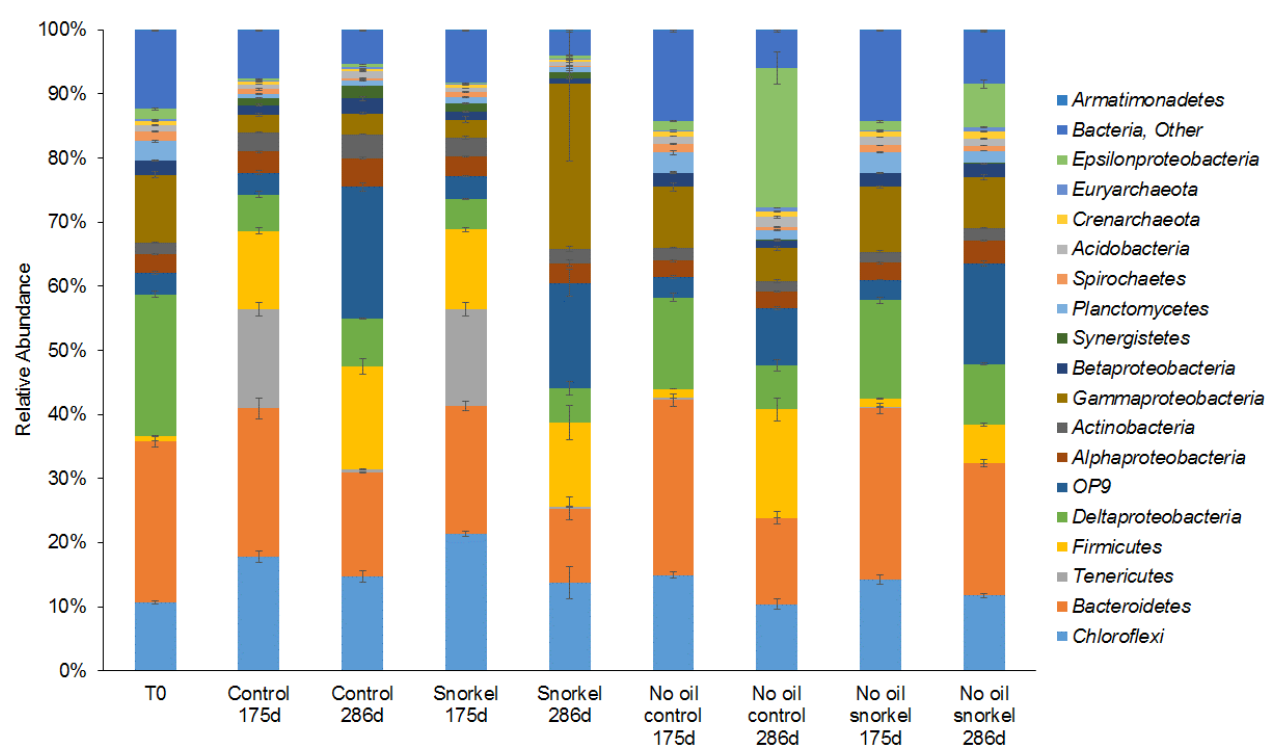


**Figure 4.** Concentration of TPH in the different in snorkel experiments and controls throughout the experimental period. Error bars represent the standard error of replicate samples.

### 3.5 Effect of electrochemical snorkels on sediment microbial communities

Thirty 16S rRNA gene amplicon libraries were generated which represented microbial communities from the bulk sediment at the start of the experiment (T0) and in the snorkel, no oil snorkel, control, and no oil control treatments following 175 and 286 days of incubation (Figure 5). The microbial composition across the 30 amplicon libraries was 98.6%  $\pm$  0.61 Bacteria (range 97.8-99.5) and 1.4%  $\pm$  0.61 Archaea (range 0.5-2.2). At the phylum level, archaeal sequences comprised a small percentage of the total microbial communities (*Euryarchaeota* 0.3%  $\pm$  0.10; *Crenarchaeota* 0.6%  $\pm$  0.17 relative abundance, Figure 5), and therefore archaeal communities were not

investigated further. The most abundant phyla in the snorkel treatment at 175 days were *Chloroflexi* (21%), *Bacterioidetes* (20%), *Tenericutes* (15%), *Firmicutes* (13%), *Deltaproteobacteria* (5%), OP9 (4%), *Alphaproteobacteria*, *Actinobacteria* and *Gammaproteobacteria* (3%), and *Betaproteobacteria*, *Synergistetes* and *Planctomycetes* (1%) (Figure 5, Supplemental Figure S1A). Successional changes in the microbial communities in the snorkel treatment were detected by day 286 with a decrease in the relative abundance of *Tenericutes* (from 15% to 0.3%), and an increase in OP9 (from 4% to 16%) and *Gammaproteobacteria* (from 3% to 26%) (Figure 5, Supplemental Figure S1B). *Deltaproteobacteria* were detected in high relative abundance in the initial sediments (T0, 22%), with a subsequent decrease in abundance in all treatments (Figure 5).



**Figure 5.** Phylum level comparison of microbial communities in River Tyne oil spill snorkel experiments. The phylum Proteobacteria were further sub-divided into Class.

Error bars represent the standard error of replicate samples (n=3, except T0 where n=6).

### 3.5.1 Putative *n*-alkane degraders

At a higher resolution within the *Chloroflexi*, the uncultured genera T78 and SHD-231 in the order *Anaerolineales* were enriched at 175 and 286 days in the snorkel and control experiments which contained oil, compared to the controls without oil and the initial sediments (Figure 6A, Supplemental Figure S2). A significant enrichment of the *Anaerolinea*, predominantly in the oil-amended treatments, suggests a role in crude oil biodegradation within the sediments. The obligately anaerobic, non-photosynthetic *Anaerolinea* (Yamada et al., 2006) have previously been implicated in oil biodegradation coupled to sulfate-reduction in River Tyne sediment microcosms (Sherry et al., 2013a), in methanogenic oil sands tailings ponds (An et al., 2013), in enrichments cultures from a low-temperature, sulfidic natural hydrocarbon seep (Savage et al., 2010) and in oil contaminated mud-flat sediments (Sanni et al., 2015). Specifically, the uncultured genus T78 have been described as saccharolytic (Yamada et al., 2006) and carbohydrate utilisers (Miura and Okabe, 2008; Yamada and Sekiguchi, 2009). T78 have been identified in samples from four biogas plants and six wastewater treatment plants used as inocula to investigate the degradation of cellulose and straw in batch cultivation tests (Sun et al., 2016). T78 have also been detected in anaerobic digesters processing sewage sludge (Ariesyady et al., 2007) and used for co-digestion of whey permeate and cow manure (Hagen et al., 2014). The increasing occurrence of the presence of members of the *Anaerolineae* in this study and previous studies suggests they may play a key role in anaerobic hydrocarbon degradation.



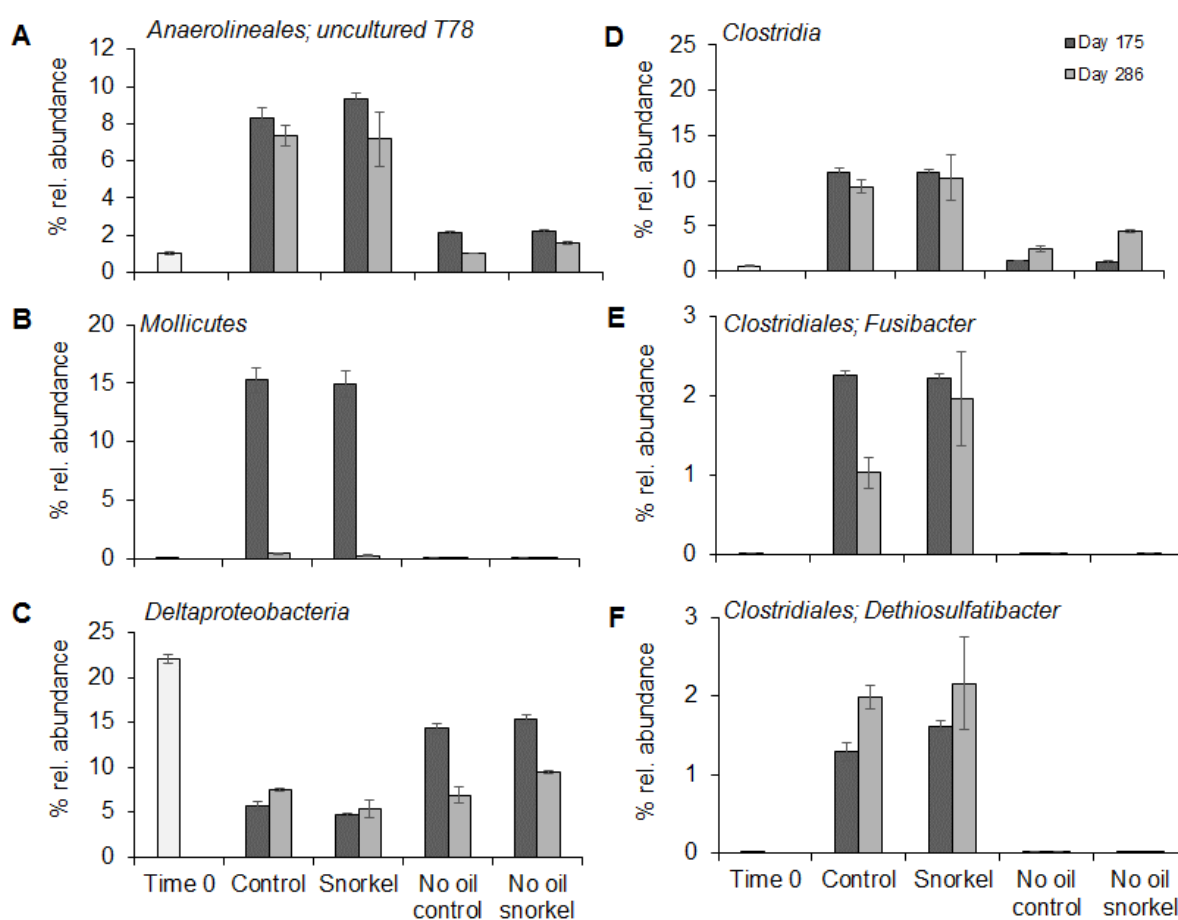
Organisms from the *Tenericutes* from within the Class *Mollicutes* were significantly enriched in oil-amended snorkel and control experiments at 175 days, compared to the oil-free controls and initial sediments (Figure 6B). The apparent absence of *Mollicutes* in the initial sediments (Time 0), the significant enrichment in only those experiments which contain oil at 175 days (where ~4 mg TPH/g sediment remained in both systems) and the absence of *Mollicutes* in the oil-free controls suggests members of the *Mollicutes* play a role in the degradation of crude oil compounds. *Mollicutes* have previously been detected in salt-marsh sediment microcosms treated with Mississippi Canyon Block 252 oil (MC252) from the Deepwater Horizon (DH) spill, where they were described as 'late responders' to the oil as their relative abundance did not increase until 3 weeks after the addition of the oil (Hagen et al., 2014). In another Deepwater Horizon study, *Mollicutes* were found in the gut of oysters taken from oil-contaminated areas (King et al., 2012), suggesting a tolerance of members of the *Mollicutes* to hydrocarbons. *Tenericutes* (*Mollicutes*) have also been found as important components of the microbial communities associated with natural hydrocarbon seeps (Skenner et al., 2016).

### 3.5.2 Sulfate-reducing bacteria (SRB) and fermentative Clostridia

The relative abundance of *Deltaproteobacteria* was highest in the time 0 sediments (22% of reads from *Deltaproteobacteria*; Figure 6C), when sulfate concentration was high ~1000mg/L. By day 175 reads from *Deltaproteobacteria* decreased to around 5% relative abundance in both treatments containing oil (control and snorkel; Figure 6C) despite differences in the levels of sulfate at this time point (control ~ 600 mg/L, snorkel ~ 10 mg/L). In treatments without oil addition the relative abundance of reads from *Deltaproteobacteria* was greater than 14-15%; Figure 6C). This suggests that some *deltaproteobacterial* SRB may be sensitive to the addition of oil as has been shown

previously (Koo et al., 2015). Moreover, previous studies of sulfate-driven oil degradation in River Tyne sediments have indicated that despite oil degradation clearly being driven by sulfate-reduction, selection for deltaproteobacterial sulfate-reducing bacteria was modest (Sherry et al., 2013a). It has been suggested that this may be due to petroleum hydrocarbon degradation in these systems being driven by sulfate-reducers acting as terminal oxidizers in a syntrophic food chain, rather than being the primary hydrocarbon oxidizers, and thermodynamic arguments have been proposed in support of this hypothesis (Head et al., 2014). Further evidence to support the possibility that sulfate-reducers are terminal oxidizers in a syntrophic food chain is the enrichment of organisms from the class *Clostridia* within the *Firmicutes* on day 175 and 286 in both oil-amended treatments (control and snorkel ~10% of reads, Figure 6D) relative to the oil-free controls and the sediments at time 0 (0.6-4% of reads, Figure 6D). Within the *Clostridiales*, the genera *Fusibacter* and *Dethiosulfatibacter* were enriched in response to oil on day 175 and 286 (control and snorkel, Figure 6E and 6F) compared to oil-free controls and the sediments at time 0. *Fusibacter* spp. have been associated with oil-producing wells (Ravot et al., 1999), sites contaminated with chlorinated solvents (Lee et al., 2011), and degradation of PAHs following the Deepwater Horizon spill (Kappell et al., 2014). Furthermore, *Fusibacter* were shown to be involved in the degradation of alkanes, crude oil and aromatics to volatile fatty acids (VFAs) in a study investigating which crude oil components contribute to oil field souring (Hasegawa et al., 2014). *Fusibacter* prefer anaerobic environments and are thiosulfate-reducers with a fermentative metabolism that can produce acetate, butyric acid, CO<sub>2</sub>, and H<sub>2</sub> from carbohydrates (Basso et al., 2009). Similarly, *Dethiosulfatibacter* are able to use thiosulfate and elemental sulfur as electron acceptors and can produce CO<sub>2</sub>, H<sub>2</sub>, acetate, and propionate from organic matter (Takii et al., 2007). Interestingly, geochemical data

suggest that in the presence of crude oil, the snorkel may promote oxidation of sulfide to elemental sulfur and/or thiosulfate, potentially explaining the selection of these organisms in treatments containing electrochemical snorkels (see section 3.7 below). Both *Fusibacter* and *Dethiosulfatibacter* are hydrogen-producing acetogenic bacteria that are potentially involved in fermenting components of oil to VFA which may be utilized by sulfate-reducing terminal oxidizers.



**Figure 6.** Relative abundance (%) of rRNA gene sequences (n=11214) from oil spill snorkel experiments at 0, 175 and 286 days. The uncultured genus T78 lineage in the order *Anaerolineales* (A), class *Mollicutes* (B), class *Deltaproteobacteria* (C), class *Clostridia* (D), genus *Fusibacter* (order *Clostridiales*) (E) and genus *Dethiosulfatibacter*

(order *Clostridiales*) (F). Error bars denote standard error (n=3, except Time 0 where n=6). Note differences in scale.

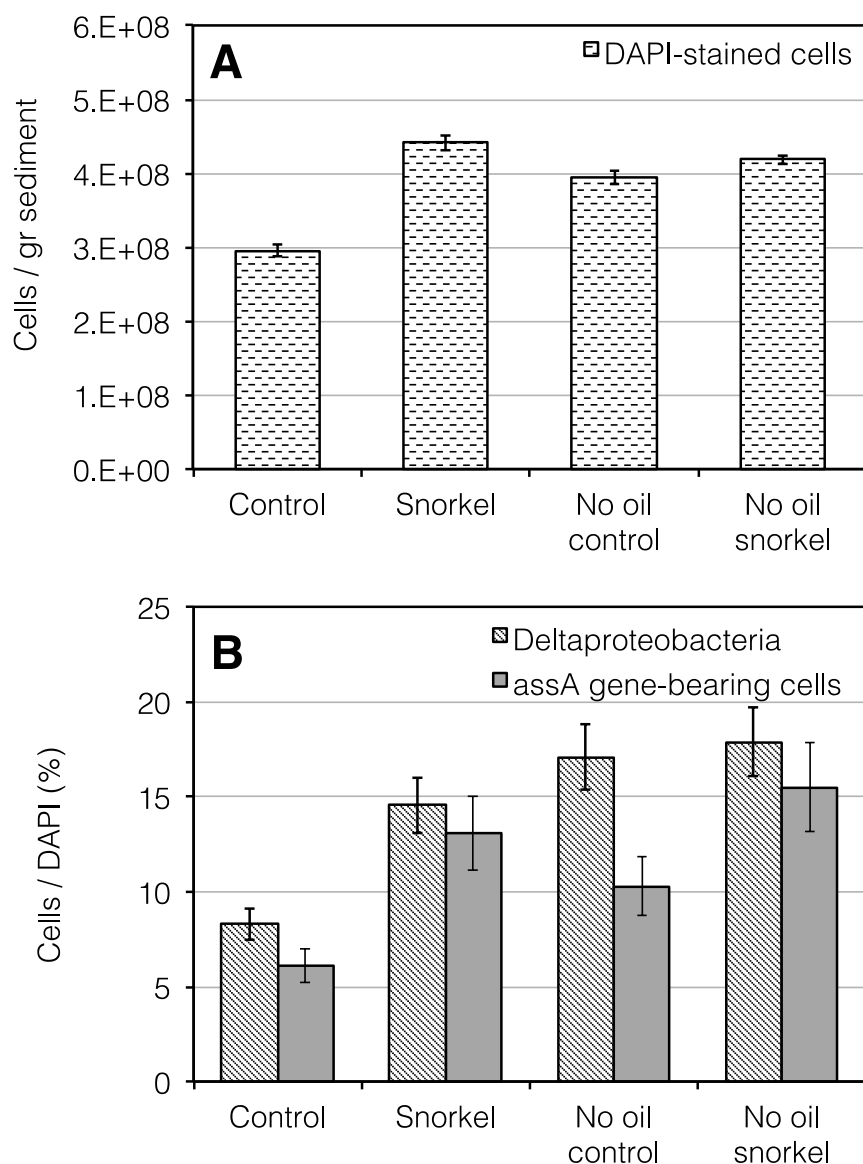
### 3.6 Effect of electrochemical snorkels on abundance of prokaryote cells, *Deltaproteobacteria* and anaerobic alkane-degrading bacteria

Consistent with the effect of electrochemical snorkels on sulfate-reduction rates and hydrocarbon degradation, the snorkel and the supplied oil were found to exert an effect on the overall abundance of prokaryote cells. After 286 days of incubation, in the oil-supplemented snorkel treatment, the abundance of DAPI-stained cells in the bulk sediment was higher ( $p=0.007$ ) than in the corresponding control ( $4.4\pm0.1 \times 10^8$  vs.  $2.9\pm0.1 \times 10^8$  cells per gram of dry sediment; Figure 7a). This corroborated the suggestion that electrochemical snorkels promoted growth-linked metabolism within the oil-treated sediment (Figure 7a). Notably, the DAPI cell counts in the bulk sediment of no-oil controls ( $3.9\pm0.1 \times 10^8$  cells per gram of dry sediment) was substantially higher than in the oil-supplemented controls with no snorkel, clearly indicating that the presence of oil adversely affected microbial activity (Figure 7a).

The trend in total cell counts was mirrored by the abundance of *Deltaproteobacteria* in the different treatments. Their higher abundance in the oil-supplemented snorkels with respect to the (oil-supplemented) controls is consistent with the observed positive effect of the snorkel on sulfate-reducing activity and alkane degradation. The trend in the *Deltaproteobacteria* was also mirrored in the relative abundance data at 286 days (cf. Figure 7b with Figure 6C).

An increasing number of studies have shown that fumarate addition is a key mechanism of alkane activation under anoxic conditions (Callaghan et al., 2010; von Netzer et al.,

2013). This reaction is catalyzed by alkylsuccinate synthase. The gene encoding the alpha subunit of alkylsuccinate synthase *assA*, can serve as a biomarker of anaerobic alkane degradation. Here, a geneCARD-FISH approach was employed to enumerate the presence and enrichment of cells carrying the *assA* gene in the different treatments, both in the bulk sediment and on the surface of the graphite snorkels. Interestingly, after 286 days of incubation, the measured concentration of cells carrying the *assA* gene was similar to the abundance of *Deltaproteobacteria*, suggesting that sulfate-reducing bacteria also played a role in direct petroleum hydrocarbon oxidation in River Tyne sediments (Sherry et al., 2013b). It remains unclear why, in no-oil treatments, the measured concentration of *Deltaproteobacteria* and cells carrying the *assA* gene was higher than in the oil-supplemented microcosms. This may result from the fact, that in the absence of snorkels the presence of oil adversely affected the autochthonous microbial populations in the sediment, as is evident from the DAPI count data and the Ion Torrent sequencing data (Figure 6C and 7A). Analysis of the biofilm growing on the surface of the snorkels indicated that cells carrying the *assA* gene were present at comparable levels ( $11 \pm 2$  vs.  $7 \pm 1$  % of total bacteria) in the oil-supplemented snorkels and the no-oil snorkels, suggesting a minor role for the electrode biofilm in the observed degradation of hydrocarbons.

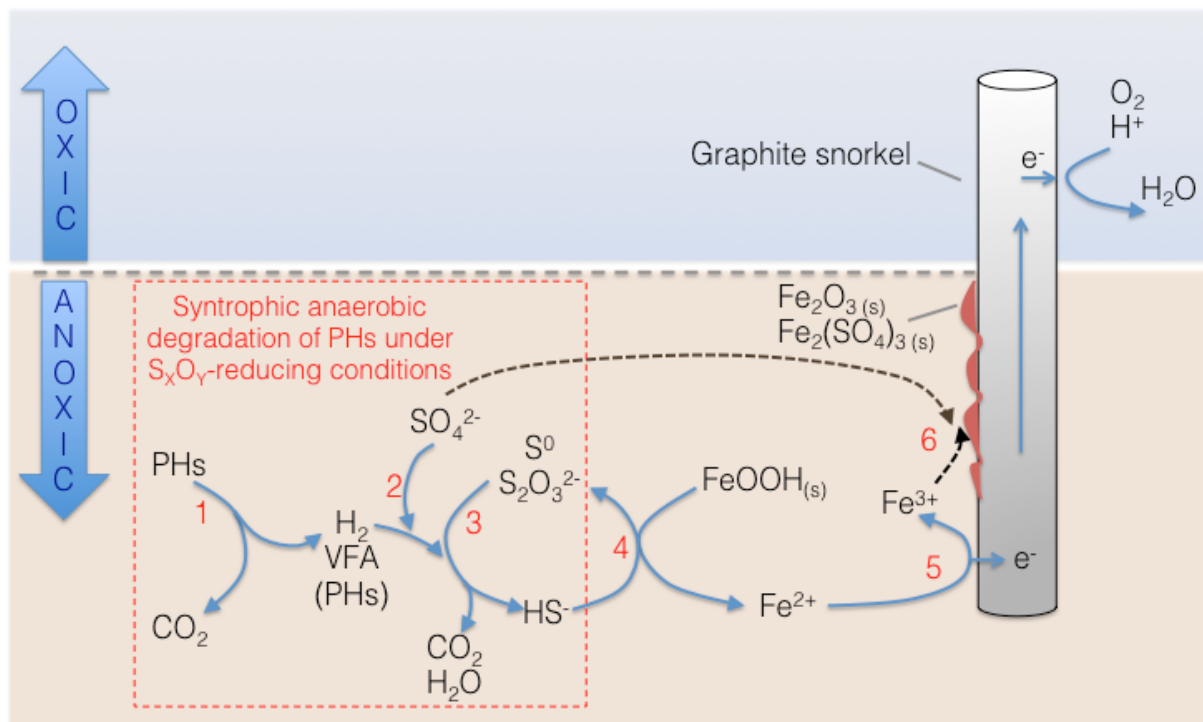


**Figure 7.** Concentration of total DAPI-stained cells in the bulk sediment of the different experiments (a). Concentration of *Deltaproteobacteria* (as detected by CARD-FISH) and of cells carrying the *assA* gene in the bulk sediment of the different treatments (as detected by geneCARD-FISH) (b). All measurements were carried out on samples taken after 286 days of incubations. Error bars represent the standard error of replicate samples.

### 3.7 *Interaction of graphite electrodes and sediment biogeochemical cycles*

This study provides evidence that bridging spatially separated redox zones (i.e., the anoxic sediment with the oxic overlying water) with an electrically conductive snorkel has a remarkable impact on a number of biogeochemical processes taking place in the bulk sediment, even at a substantial distance from the surface of the conductive element (i.e., the snorkel). The most striking finding was the acceleration of sulfate reduction in the presence of an electrochemical “snorkel”, with this process being apparently coupled to the formation of oxidized iron species on the surface of the snorkel. Possibly, Fe(III) species accumulating at the surface of the rod derived from the reductive dissolution of iron minerals (e.g., Fe(III) (hydr)oxides) in the sediment into Fe(II), driven by biogenically produced hydrogen sulfide (Aller and Rude, 1988), followed by the (bio)electrochemical re-oxidation of Fe(II) to Fe(III) at the electrode surface (Figure 8). It is worth noting, that GC-MS analyses of sediment extracts indicated the presence of substantial amounts of elemental sulfur in the River Tyne sediments (data not shown), suggesting that this compound was a likely product of hydrogen sulfide oxidation, and that this reaction most probably also occurred under “natural” conditions. Interestingly, thermodynamic calculations indicated that sulfide oxidation to elemental sulfur coupled to Fe(III) reduction to Fe(II), under conditions relevant to those occurring in the snorkel experiments (i.e., pH, concentration of reactants and products), is extremely sensitive to Fe(II) concentration and becomes endergonic at Fe(II) concentrations higher than  $10^{-5}$  M (Figure 9). This provides an indication that, by scavenging Fe(II), the snorkel may affect hydrogen sulfide accumulation and in turn sulfate reduction.

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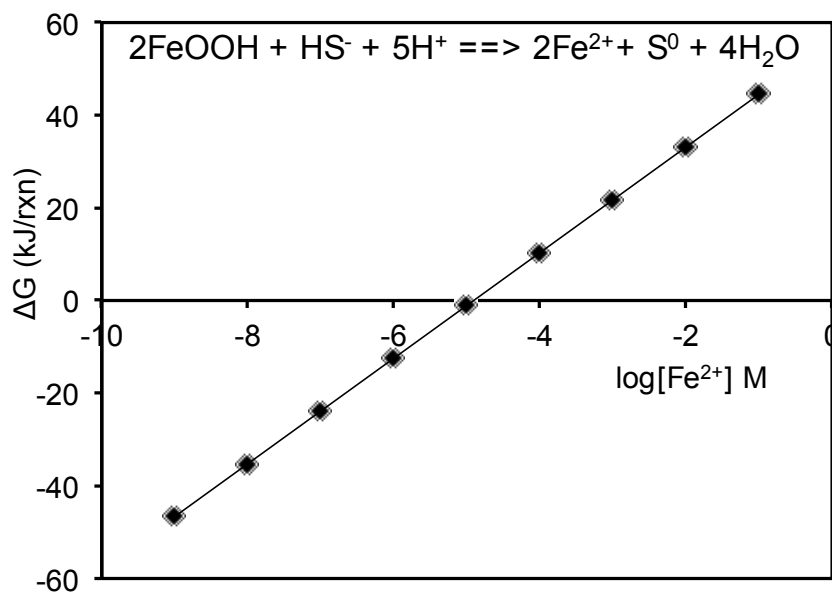
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**Figure 8.** Tentative model depicting the effects of an electrochemical snorkel on chemical and biological reactions taking place in sediments contaminated by petroleum hydrocarbons. Legend: (1) Fermentative degradation of Petroleum Hydrocarbons (PHs), tentatively catalyzed by *Clostridia*; (2) Sulfate reduction coupled to oxidation of fermentation products or petroleum hydrocarbons, tentatively catalyzed by *Deltaproteobacteria*; (3) Sulfur / thiosulfate reduction coupled to oxidation of fermentation products; (4) Abiotic reduction of Fe(III)hydroxides coupled to oxidation of sulfide to sulfur / thiosulfate; (5) Biotic/abiotic oxidation of Fe<sup>2+</sup> coupled to electrode reduction; (6) Precipitation of poorly soluble Fe(III) compounds.





**Figure 9.** Influence of  $\text{Fe}^{2+}$  concentration on the Gibbs free energy change of Fe(III) (hydr)oxide reduction by hydrogen sulfide.

#### 4. Conclusions

The results of this study provide an additional line of evidence that a microbial electrochemical snorkel (i.e., a single graphite electrode half-buried in anoxic sediments and half exposed to oxygenated overlying water) has a remarkable impact on biogeochemical redox processes taking place in the bulk sediment. The most noticeable effect was observed on sulfate reduction, which was substantially accelerated by the presence of the snorkel compared to snorkel-free controls, while methanogenesis was apparently suppressed. Accumulation of oxidized iron species on the electrode surface, along with thermodynamic calculations suggested that the occurrence of a sulfide-driven iron redox cycle was triggered by the presence of the snorkel. Acceleration of sulfate reduction corresponded to a slightly enhanced removal of petroleum hydrocarbons may be facilitated by removal of toxic sulfide linked to reduction of iron

oxides in the sediment. This process may enhance overall sulfide oxidation with the Fe(II) generated from sulfide oxidation being removed by oxidation at the snorkel/electrode surface, maintaining a low Fe(II) concentration that provides a thermodynamic driver favoring sulfide oxidation, while replenishing Fe(III) as an oxidant, sustaining the process over extended time periods.

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## **Author contribution**

C.C.V. set up and monitored the oil-spill snorkel experiments. B.M., E.F., and S.R. performed and analyzed FISH experiments. S.I. developed the GC-MS method and contributed to the interpretation of chemical data. A.M and S.K performed and analyzed XRD experiments. A.S. and O.K.M. and I.M.H performed NGS analysis. E.V. and K.R. contributed the analysis and interpretation of Fe and S biogeochemical cycles. F.A conceived the experimental plan. All authors contributed to writing of the manuscript. F.A., S.R., K.R. and I.M.H. were co-Investigators on the Kill-Spill Project.

## References

- Aitken, C.M., Jones, D.M., Maguire, M.J., Gray, N.D., Sherry, A., Bowler, B.F.J., Ditchfield, A.K., Larter, S.R., Head, I.M., 2013. Evidence that crude oil alkane activation proceeds by different mechanisms under sulfate-reducing and methanogenic conditions. *Geochim. Cosmochim. Acta* 109, 162–174. doi:10.1016/j.gca.2013.01.031
- Aller, R.C., Rude, P.D., 1988. Complete oxidation of solid phase sulfides by manganese and bacteria in anoxic marine sediments. *Geochim. Cosmochim. Acta* 52, 751–765. doi:10.1016/0016-7037(88)90335-3
- An, D., Brown, D., Chatterjee, I., Dong, X., Ramos-Padron, E., Wilson, S., Bordenave, S., Caffrey, S.M., Gieg, L.M., Sensen, C.W., Voordouw, G., Scherer, S., 2013. Microbial community and potential functional gene diversity involved in anaerobic hydrocarbon degradation and methanogenesis in an oil sands tailings pond 1. *Genome* 56, 612–618. doi:10.1139/gen-2013-0083
- Ariesyady, H.D., Ito, T., Okabe, S., 2007. Functional bacterial and archaeal community structures of major trophic groups in a full-scale anaerobic sludge digester. *Water Res.* 41, 1554–1568. doi:10.1016/j.watres.2006.12.036
- Bargiela, R., Mapelli, F., Rojo, D., Chouaia, B., Tornés, J., Borin, S., Richter, M., Del Pozo, M. V., Cappello, S., Gertler, C., Genovese, M., Denaro, R., Martínez-Martínez, M., Fodelianakis, S., Amer, R.A., Bigazzi, D., Han, X., Chen, J., Chernikova, T.N., Golyshina, O. V., Mahjoubi, M., Jaouanil, A., Benzha, F., Magagnini, M., Hussein, E., Al-Horani, F., Cherif, A., Blaghen, M., Abdel-Fattah, Y.R., Kalogerakis, N., Barbas, C., Malkawi, H.I., Golyshin, P.N., Yakimov, M.M., Daffonchio, D., Ferrer, M., 2015. Bacterial population and biodegradation potential in chronically crude oil-contaminated marine sediments are strongly linked to temperature. *Sci. Rep.* 5, 11651.

714       doi:10.1038/srep11651

715 Barra Caracciolo, A., Grenni, P., Cupo, C., Rossetti, S., 2005. In situ analysis of native  
716       microbial communities in complex samples with high particulate loads. *FEMS*  
717       *Microbiol. Lett.* 253, 55–58. doi:10.1016/j.femsle.2005.09.018

718 Basso, O., Lascourreges, J.-F., Le Borgne, F., Le Goff, C., Magot, M., 2009. Characterization  
719       by culture and molecular analysis of the microbial diversity of a deep subsurface  
720       gas storage aquifer. *Res. Microbiol.* 160, 107–116.  
721       doi:10.1016/j.resmic.2008.10.010

722 Bellagamba, M., Cruz Viggi, C., Ademollo, N., Rossetti, S., Aulenta, F., 2016. Electrolysis-  
723       driven bioremediation of crude oil-contaminated marine sediments. *N. Biotechnol.*  
724       doi:10.1016/j.nbt.2016.03.003

725 Borole, A.P., Reguera, G., Ringeisen, B., Wang, Z.-W., Feng, Y., Kim, B.H., 2011.  
726       Electroactive biofilms: Current status and future research needs. *Energy Environ.*  
727       *Sci.* 4, 4813. doi:10.1039/c1ee02511b

728 Callaghan, A. V., Davidova, I.A., Savage-Ashlock, K., Parisi, V.A., Gieg, L.M., Suflita, J.M.,  
729       Kukor, J.J., Wawrik, B., 2010. Diversity of benzyl- and alkylsuccinate synthase genes  
730       in hydrocarbon-impacted environments and enrichment cultures. *Environ. Sci.*  
731       *Technol.* 44, 7287–7294. doi:10.1021/es1002023

732 Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K.,  
733       Fierer, N., Peña, A.G., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T., Knights, D.,  
734       Koenig, J.E., Ley, R.E., Lozupone, C.A., McDonald, D., Muegge, B.D., Pirrung, M.,  
735       Reeder, J., Sevinsky, J.R., Turnbaugh, P.J., Walters, W.A., Widmann, J., Yatsunenko, T.,  
736       Zaneveld, J., Knight, R., 2010a. QIIME allows analysis of high-throughput community  
737       sequencing data. *Nat. Methods* 7, 335–336. doi:10.1038/nmeth.f.303

738 Caporaso, J.G., Bittinger, K., Bushman, F.D., DeSantis, T.Z., Andersen, G.L., Knight, R.,

2010b. PyNAST: a flexible tool for aligning sequences to a template alignment.  
 Bioinformatics 26, 266–267. doi:10.1093/bioinformatics/btp636

Conrad, R., Schink, B., Phelps, T.J., 1986. Thermodynamics of H<sub>2</sub>-consuming and H<sub>2</sub>-  
 producing metabolic reactions in diverse methanogenic environments under in situ  
 conditions. FEMS Microbiol. Lett. 38, 353–360. doi:10.1016/0378-1097(86)90013-  
 3

Daghighi, M., Vaiopoulou, E., Patil, S.A., Suárez-Suárez, A., Head, I.M., Franzetti, A., Rabaey,  
 K., 2016. Anodes stimulate anaerobic toluene degradation via sulfur cycling in  
 marine sediments. Appl. Environ. Microbiol. 82, 297–307. doi:10.1128/AEM.02250-  
 15

DeSantis, T.Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E.L., Keller, K., Huber, T.,  
 Dalevi, D., Hu, P., Andersen, G.L., 2006. Greengenes, a Chimera-Checked 16S rRNA  
 Gene Database and Workbench Compatible with ARB. Appl. Environ. Microbiol. 72,  
 5069–5072. doi:10.1128/AEM.03006-05

Edgar, R.C., 2010. Search and clustering orders of magnitude faster than BLAST.  
 Bioinformatics 26, 2460–2461. doi:10.1093/bioinformatics/btq461

Fazi, S., Aulenta, F., Majone, M., Rossetti, S., 2008. Improved quantification of  
 Dehalococcoides species by fluorescence in situ hybridization and catalyzed  
 reporter deposition. Syst. Appl. Microbiol. 31, 62–67.  
 doi:10.1016/j.syapm.2007.11.001

Genovese, M., Crisafi, F., Denaro, R., Cappello, S., Russo, D., Calogero, R., Santisi, S.,  
 Catalfamo, M., Modica, A., Smedile, F., Genovese, L., Golyshin, P.N., Giuliano, L.,  
 Yakimov, M.M., 2014. Effective bioremediation strategy for rapid in situ cleanup of  
 anoxic marine sediments in mesocosm oil spill simulation. Front. Microbiol. 5.  
 doi:10.3389/fmicb.2014.00162

764 Gray, N.D., Sherry, A., Grant, R.J., Rowan, A.K., Hubert, C.R.J., Callbeck, C.M., Aitken, C.M.,  
 765 Jones, D.M., Adams, J.J., Larter, S.R., Head, I.M., 2011. The quantitative significance of  
 766 Syntrophaceae and syntrophic partnerships in methanogenic degradation of crude  
 767 oil alkanes. *Environ. Microbiol.* 13, 2957–2975. doi:10.1111/j.1462-  
 768 2920.2011.02570.x

769 Haas, B.J., Gevers, D., Earl, A.M., Feldgarden, M., Ward, D. V., Giannoukos, G., Ciulla, D.,  
 770 Tabbaa, D., Highlander, S.K., Sodergren, E., Methé, B., DeSantis, T.Z., Petrosino, J.F.,  
 771 Knight, R., Birren, B.W., 2011. Chimeric 16S rRNA sequence formation and detection  
 772 in Sanger and 454-pyrosequenced PCR amplicons. *Genome Res.* 21, 494–504.  
 773 doi:10.1101/gr.112730.110

774 Hagen, L.H., Vivekanand, V., Linjordet, R., Pope, P.B., Eijsink, V.G.H., Horn, S.J., 2014.  
 775 Microbial community structure and dynamics during co-digestion of whey  
 776 permeate and cow manure in continuous stirred tank reactor systems. *Bioresour.*  
 777 *Technol.* 171, 350–359. doi:10.1016/j.biortech.2014.08.095

778 Hasegawa, R., Toyama, K., Miyanaga, K., Tanji, Y., 2014. Identification of crude-oil  
 779 components and microorganisms that cause souring under anaerobic conditions.  
 780 *Appl. Microbiol. Biotechnol.* 98, 1853–1861. doi:10.1007/s00253-013-5107-3

781 Head, I.M., 1999. Recovery and analysis of ribosomal RNA sequences from the  
 782 environment, in: Edwards, C. (Ed.), *Environmental Monitoring of Bacteria*. Humana  
 783 Press, Totowa, New Jersey, pp. 139–174.

784 Head, I.M., Gray, N.D., Larter, S.R., 2014. Life in the slow lane; biogeochemistry of  
 785 biodegraded petroleum containing reservoirs and implications for energy recovery  
 786 and carbon management. *Front. Microbiol.* 5, 566. doi:10.3389/fmicb.2014.00566

787 Jones, D.M., Head, I.M., Gray, N.D., Adams, J.J., Rowan, A.K., Aitken, C.M., Bennett, B.,  
 788 Huang, H., Brown, A., Bowler, B.F.J., Oldenburg, T., Erdmann, M., Larter, S.R., 2008.

789 Crude-oil biodegradation via methanogenesis in subsurface petroleum reservoirs.  
790 Nature 451, 176–180. doi:10.1038/nature06484

791 Kappell, A.D., Wei, Y., Newton, R.J., Van Nostrand, J.D., Zhou, J., McLellan, S.L., Hristova,  
792 K.R., 2014. The polycyclic aromatic hydrocarbon degradation potential of Gulf of  
793 Mexico native coastal microbial communities after the Deepwater Horizon oil spill.  
794 Front. Microbiol. 5, 205. doi:10.3389/fmicb.2014.00205

795 King, G.M., Judd, C., Kuske, C.R., Smith, C., 2012. Analysis of Stomach and Gut  
796 Microbiomes of the Eastern Oyster (*Crassostrea virginica*) from Coastal Louisiana,  
797 USA. PLoS One 7, e51475. doi:10.1371/journal.pone.0051475

798 Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., Glöckner, F.O.,  
799 2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and  
800 next-generation sequencing-based diversity studies. Nucleic Acids Res. 41, e1.  
801 doi:10.1093/nar/gks808

802 Koo, H., Mojib, N., Huang, J.P., Donahoe, R.J., Bej, A.K., 2015. Bacterial community shift in  
803 the coastal Gulf of Mexico salt-marsh sediment microcosm in vitro following  
804 exposure to the Mississippi Canyon Block 252 oil (MC252). 3 Biotech 5, 379–392.  
805 doi:10.1007/s13205-014-0233-x

806 Kuivila, K.M., Murray, J.W., Devol, A.H., Novelli, P.C., 1989. Methane production, sulfate  
807 reduction and competition for substrates in the sediments of Lake Washington.  
808 Geochim. Cosmochim. Acta 53, 409–416. doi:10.1016/0016-7037(89)90392-X

809 Lee, J., Lee, T.K., Löffler, F.E., Park, J., 2011. Characterization of microbial community  
810 structure and population dynamics of tetrachloroethene-dechlorinating tidal  
811 mudflat communities. Biodegradation 22, 687–698. doi:10.1007/s10532-010-  
812 9429-x

813 Lovley, D.R., Klug, M.J., 1986. Model for the distribution of sulfate reduction and

methanogenesis in freshwater sediments. *Geochim. Cosmochim. Acta* 50, 11–18.  
doi:10.1016/0016-7037(86)90043-8

Lovley, D.R., Nevin, K.P., 2011. A shift in the current: New applications and concepts for  
microbe-electrode electron exchange. *Curr. Opin. Biotechnol.*  
doi:10.1016/j.copbio.2011.01.009

Matturro, B., Frascadore, E., Cappello, S., Genovese, M., Rossetti, S., 2016. In situ  
detection of alkB2 gene involved in *Alcanivorax borkumensis* SK2T hydrocarbon  
biodegradation. *Mar. Pollut. Bull.* 110, 378–382.  
doi:10.1016/j.marpolbul.2016.06.038

Meckenstock, R.U., Safinowski, M., Griebler, C., 2004. Anaerobic degradation of polycyclic  
aromatic hydrocarbons, in: *FEMS Microbiology Ecology*. pp. 27–36.  
doi:10.1016/j.femsec.2004.02.019

Miura, Y., Okabe, S., 2008. Quantification of cell specific uptake activity of microbial  
products by uncultured *Chloroflexi* by microautoradiography combined with  
fluorescence in situ hybridization. *Environ. Sci. Technol.* 42, 7380–6.  
doi:10.1021/es800566e

Morris, J.M., Jin, S., Crimi, B., Pruden, A., 2009. Microbial fuel cell in enhancing anaerobic  
biodegradation of diesel. *Chem. Eng. J.* doi:10.1016/j.cej.2008.05.028

O’Sullivan, L.A., Roussel, E.G., Weightman, A.J., Webster, G., Hubert, C.R.J., Bell, E., Head, I.,  
Sass, H., Parkes, R.J., 2015. Survival of *Desulfotomaculum* spores from estuarine  
sediments after serial autoclaving and high-temperature exposure. *ISME J.* 9, 922–  
33. doi:10.1038/ismej.2014.190

Oremland, R.S., Polcin, S., 1982. Methanogenesis and sulfate reduction: competitive and  
noncompetitive substrates in estuarine sediments. *Appl. Environ. Microbiol.* 44,  
1270–1276. doi:10.1016/0198-0254(83)90262-5



839 Oremland, R.S., Taylor, B.F., 1978. Sulfate reduction and methanogenesis in marine  
840 sediments. *Geochim. Cosmochim. Acta* 42, 209–214. doi:10.1016/0016-  
841 7037(78)90133-3

842 Paulo, L.M., Stams, A.J.M., Sousa, D.Z., 2015. Methanogens, sulphate and heavy metals: a  
843 complex system. *Rev. Environ. Sci. Bio/Technology* 14, 537–553.  
844 doi:10.1007/s11157-015-9387-1

845 Quince C, Lanzen A, Davenport R, Turnbaugh P (2011) Removing noise from  
846 pyrosequenced amplicons. *BMC Bioinform* 12, 38. doi:10.1186/1471-2105-12-38

847 Ravot, G., Magot, M., Fardeau, M.-L., Patel, B.K., Thomas, P., Garcia, J.-L., 1999.  
848 *Fusibacter paucivorans* gen. nov., sp. nov., an anaerobic, thiosulfate-reducing  
849 bacterium from an oil-producing well. *Int J Syst Bacteriol* 49, 1141–1147.  
850 doi:10.1099/00207713-49-3-1141

851 Reis, M., Almeida, J.S., Lemos, P.C., Carrondo, M.J., 1992. Effect of hydrogen sulfide on  
852 growth of sulfate reducing bacteria. *Biotechnol. Bioeng.* 40, 593–600.  
853 doi:10.1002/bit.260400506

854 Sanni, G.O., Coulon, F., McGenity, T.J., 2015. Dynamics and distribution of bacterial and  
855 archaeal communities in oil-contaminated temperate coastal mudflat mesocosms.  
856 *Environ. Sci. Pollut. Res.* 22, 15230–15247. doi:10.1007/s11356-015-4313-1

857 Savage, K.N., Krumholz, L.R., Gieg, L.M., Parisi, V.A., Suflita, J.M., Allen, J., Philp, R.P.,  
858 Elshahed, M.S., 2010. Biodegradation of low-molecular-weight alkanes under  
859 mesophilic, sulfate-reducing conditions: metabolic intermediates and community  
860 patterns. *FEMS Microbiol. Ecol.* 72, 485–495. doi:10.1111/j.1574-  
861 6941.2010.00866.x

862 Sherry, A., Grant, R.J., Aitken, C.M., Jones, D.M., Head, I.M., Gray, N.D., 2014. Volatile  
863 hydrocarbons inhibit methanogenic crude oil degradation. *Front. Microbiol.* 5.

doi:10.3389/fmicb.2014.00131

Sherry, A., Gray, N.D., Ditchfield, A.K., Aitken, C.M., Jones, D.M., Röling, W.F.M., Hallmann, C., Larter, S.R., Bowler, B.F.J., Head, I.M., 2013a. Anaerobic biodegradation of crude oil under sulphate-reducing conditions leads to only modest enrichment of recognized sulphate-reducing taxa. *Int. Biodeterior. Biodegrad.* 81, 105–113. doi:10.1016/j.ibiod.2012.04.009

Sherry, A., Gray, N.D., Ditchfield, A.K., Aitken, C.M., Jones, D.M., Röling, W.F.M., Hallmann, C., Larter, S.R., Bowler, B.F.J., Head, I.M., 2013b. Anaerobic biodegradation of crude oil under sulphate-reducing conditions leads to only modest enrichment of recognized sulphate-reducing taxa. *Int. Biodeterior. Biodegrad.* 81, 105–113. doi:10.1016/j.ibiod.2012.04.009

Skenneron, C.T., Haroon, M.F., Briegel, A., Shi, J., Jensen, G.J., Tyson, G.W., Orphan, V.J., 2016. Phylogenomic analysis of Candidatus “Izimaplasma” species: free-living representatives from a Tenericutes clade found in methane seeps. *ISME J.* 10, 2679–2692. doi:10.1038/ismej.2016.55

Spormann, A.M., Widdel, F., 2000. Metabolism of alkylbenzenes, alkanes, and other hydrocarbons in anaerobic bacteria. *Biodegradation* 11, 85–105. doi:10.1023/A:1011122631799

Sun, L., Liu, T., Müller, B., Schnürer, A., 2016. The microbial community structure in industrial biogas plants influences the degradation rate of straw and cellulose in batch tests. *Biotechnol. Biofuels* 9, 128. doi:10.1186/s13068-016-0543-9

Takii, S., Hanada, S., Tamaki, H., Ueno, Y., Sekiguchi, Y., Ibe, A., Matsuura, K., 2007. *Dethiosulfatibacter aminovorans* gen. nov., sp. nov., a novel thiosulfate-reducing bacterium isolated from coastal marine sediment via sulfate-reducing enrichment with Casamino acids. *Int. J. Syst. Evol. Microbiol.* 57, 2320–2326.

doi:10.1099/ijs.0.64882-0

Viggi, C.C., Presta, E., Bellagamba, M., Kaciulis, S., Balijepalli, S.K., Zanaroli, G., Papini, M.P., Rossetti, S., Aulenta, F., 2015. The “Oil-Spill Snorkel”: An innovative bioelectrochemical approach to accelerate hydrocarbons biodegradation in marine sediments. *Front. Microbiol.* 6. doi:10.3389/fmicb.2015.00881

von Netzer, F., Pilloni, G., Kleindienst, S., Krüger, M., Knittel, K., Gründger, F., Luedersa, T., 2013. Enhanced gene detection assays for fumarate-adding enzymes allow uncovering of anaerobic hydrocarbon degraders in terrestrial and marine systems. *Appl. Environ. Microbiol.* 79, 543–552. doi:10.1128/AEM.02362-12

Wang, Q., Garrity, G.M., Tiedje, J.M., Cole, J.R. 2007. Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* 73, 5261-5267. doi:10.1128/AEM.00062-07

Wang, H., Ren, Z.J., 2013. A comprehensive review of microbial electrochemical systems as a platform technology. *Biotechnol. Adv.* 31, 1796–1807. doi:10.1016/j.biotechadv.2013.10.001

Yamada, T., Sekiguchi, Y., 2009. Cultivation of uncultured chloroflexi subphyla: significance and ecophysiology of formerly uncultured chloroflexi “subphylum i” with natural and biotechnological relevance. *Microbes Environ.* 24, 205–216. doi:10.1264/jsme2.ME09151S

Yamada, T., Sekiguchi, Y., Hanada, S., Imachi, H., 2006. *Anaerolinea thermolimosa* sp. nov., *Levilinea saccharolytica* gen. nov., sp. nov. and *Leptolinea tardivitalis* gen. nov., sp. nov., novel filamentous anaerobes, and description of the new classes *Anaerolineae* classis nov. and *Caldilineae* classis nov. in the. *Int. J. Syst. Evol. Microbiol.* 56, 1331–1340. doi:10.1099/ijs.0.64169-0

Yan, F., Reible, D., 2014. Electro-bioremediation of contaminated sediment by electrode

914 enhanced capping. J. Environ. Manage. 155, 154–161.  
915 doi:10.1016/j.jenvman.2015.03.023  
916 Zhang, F., Cheng, S., Pant, D., Bogaert, G. Van, Logan, B.E., 2009. Power generation using  
917 an activated carbon and metal mesh cathode in a microbial fuel cell. Electrochem.  
918 commun. 11, 2177–2179. doi:10.1016/j.elecom.2009.09.024  
919